

UNIVERSIDADE DE BRASÍLIA  
FACULDADE DE CIÊNCIAS DA SAÚDE

FELIPE DE QUEIROZ PIRES

DEVELOPMENT OF LIPID NANOPARTICLES LOADING THYMOL AND *Lippia  
origanoides* ESSENTIAL OIL IN INCLUSION COMPLEXES WITH CYCLODEXTRIN

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Dissertação apresentada como requisito parcial para a obtenção do Título de Mestre em Ciências Farmacêuticas pelo Programa de Pós-Graduação em Ciências da Farmacêuticas da Universidade de Brasília.

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Development of lipid nanoparticles loading thymol and Lippia origanoides essential oil in inclusion complexes with cyclodextrin

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*“Fairy tales are more than true: not because they tell us that dragons exist, but because they tell us that dragons can be beaten.”*

*(Neil Gaiman)*

## RESUMO

O objetivo desse estudo foi a confecção de carreadores lipídicos nanoestruturados utilizando como componente ativo a forma complexada do timol e do óleo essencial de *Lippia origanoides* em ciclodextrinas, para utilização em sistemas de administração tópica com potencial para tratamento de distúrbios de pele. A primeira parte do trabalho consistiu na obtenção de um método analítico cromatográfico seletivo para determinação de timol em estudos *in vitro* de permeação na pele. O método desenvolvido apresentou elevada capacidade de recuperação do timol das camadas da pele e provou ser adequado segundo todos os parâmetros de validação avaliados. A segunda parte consistiu em um estudo de pré-formulação utilizando desenho experimental de mistura visando avaliar a compatibilidade dos componentes da formulação do nanocarreador lipídico. A partir desses estudos foram definidos os excipientes a serem usados na fase seguinte, excluindo o tensoativo taurodeoxicolato de sódio que apresentou ação sinérgica com outros componentes reduzindo a estabilidade do timol. A terceira parte do trabalho consistiu na obtenção de complexos de inclusão de timol e óleo essencial a partir de uma seleção diferentes ciclodextrinas e métodos de obtenção de complexos de inclusão em estado sólido. Os resultados mostraram que a 2-hidroxipropil- $\beta$ -ciclodextrina foi capaz de formar um complexo mais estável com o timol e que o processo de liofilização conseguiu produzir um material com maiores evidências de encapsulação, tanto para o timol, quanto para o óleo essencial, com maior estabilidade térmica e melhor solubilidade. Na parte final do trabalho, carreadores lipídicos nanoestruturados foram produzidos a partir da forma livre e complexada de timol e óleo essencial obtidos por liofilização. Comparando com a solução padrão dos ativos, todos os nanocarreadores foram capazes de controlar a liberação e a permeação do timol nas camadas da pele, sendo os nanocarreadores confeccionados com o complexo de inclusão capazes de reter completamente o componente ativo nas camadas iniciais da pele. Assim, os sistemas de liberação desenvolvidos apresentaram estabilidade e capacidade de controlar a liberação do timol e óleo essencial evidenciando grande potencial de aplicação farmacêutica.

**Palavras-chave:** Timol, *Lippia origanoides*, Ciclodextrina, Complexos de inclusão Carreadores lipídicos nanoestruturados.

## ABSTRACT

The objective of this study was the preparation of nanostructured lipid carriers using as an active component the complexed form of thymol and the essential oil of *Lippia origanoides* in cyclodextrins for use in topical delivery systems with potential for the treatment of skin disorders. The first part of the work consisted of obtaining a selective chromatographic analytical method for the determination of thymol *in vitro* studies of permeation in the skin. The developed method presented a high recovery capacity of thymol from the skin layers and proved to be adequate according to all the validation parameters tested. The second part consisted of a preformulation study using experimental mixing design to evaluate the compatibility of components of the lipid nanocarrier formulation. From these studies were defined the excipients to be used in the next phase, excluding the sodium taurodeoxycholate surfactant that presented synergistic action with other components reducing the thymol stability. The third part of the work consisted of obtaining inclusion complexes of thymol and essential oil from a selection of different cyclodextrins and methods of obtaining solid inclusion complexes. The results showed that 2-hydroxypropyl- $\beta$ -cyclodextrin was able to form a more stable complex with thymol and that the lyophilization process was able to produce a material with higher evidence of encapsulation with both thymol and essential oil, with higher thermal stability and better solubility. In the final part of the work, nanostructured lipid carriers were produced from the free and complexed form of thymol and essential oil obtained by lyophilization. Compared with the solution containing the active compounds, all nanocarriers were able to control the release and permeation of thymol in the skin layers, and the nanocarriers made with the inclusion complex were able to completely retain the active component in the initial layers of the skin. Thus, the delivery systems developed showed stability and ability to control the release of thymol and essential oil evidencing great potential for pharmaceutical application.

**Keywords:** Thymol, *Lippia origanoides*, Cyclodextrin, Inclusion Complexes, Nanostructured Lipid Carriers.



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## LIST OF ABBREVIATIONS

CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CD	Cyclodextrin
CE	Complexation efficiency
CNPQ	Conselho Nacional de Desenvolvimento Científico e Tecnológico
DL	Drug Loading
DP	Dissolution profile
DrTG	First derivative of TGA
DSC	Differential scanning calorimetry
DTA	Differential thermal analysis
EE	Encapsulation Efficiency
EO	Essential Oil
FAP-DF	Fundação de Apoio a Pesquisa do Distrito Federal
FD	Freeze Drying
FDA	Food and drug administration
FF	Filtered Fraction
FTIR	Fourier Transformed infrared spectroscopy
HPLC	High profile liquid chromatography
HP $\beta$ CD	Hydroxypropyl- $\beta$ -cyclodextrin
LC	Soybean lecithin
LOD	Limit of detection
LOQ	Limit of quantification
NLC	Nanostructured lipid carrier
P80	Polysorbate 80
PDI	Polydispersity index
PM	Physical mixture
RE	Rotary Evaporation
RS	Remain skin
RT	Retention time
SA	Stearic acid
SC	Stratum corneum
SCCO <sub>2</sub>	Supercritical CO <sub>2</sub>

SD	Spray Drying
SEM	Scanning electron microscopy
TAU	Sodium Taurodeoxycolat
TC	Theoretical concentration
TD	Total drug
TGA	Termogravimetry
TL	Total lipid
TML	Thymol
UV	Ultraviolet

## SUMMARY

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## CHAPTER 1 - JUSTIFICATION AND LITERATURE REVIEW

### 1.1 INTRODUCTION

Thymol (TML) is a monoterpene presents as a majority compound in the essential oils of several plants. This molecule, in isolated form or in natural extracts, has a large spectra of pharmacological activities, such as antioxidant, antimicrobial, cicatrizing and protective action against damages of UV radiation (KAVOOSI; DADFAR; PURFARD, 2013; MARCHESE et al., 2016; PEREZ-VASQUEZ et al., 2011; RIELLA et al., 2012). Despite the vast therapeutic potential of TML, its pharmaceutical use, particularly in skin topical treatments, is practically unexplored.

The first step to enable the TML administration to the skin is to find a selective analytical method capable of quantifying the drug extracted from the skin, since this complex organ has many contaminants that may interfere with the analysis. Although literature has already reported some chromatographic methods for TML assaying (CHEN; CHEN, 2012; HAJIMEHDIPOOR et al., 2010; LI; YUAN; SU, 2006; SOLINAS; GESSA; DELITALA, 1981), none of them evaluated drug selectivity in the presence of possible skin interferents.

The unfavorable physicochemical characteristics of TML, mainly its volatility, light sensitivity and low water solubility is a decisive issue which needs to be overcome for its therapeutic exploitation. In this sense, TML complexation with cyclodextrins (CDs) has proved to be a very promising approach (ABARCA et al., 2015; BETHANIS et al., 2013; LOCCI et al., 2004; MARRETO et al., 2008; MULINACCI et al., 1996; RASSU et al., 2014; TAO et al., 2014).

Moreover, for TML topical use, it is also necessary to insert this drug in a controlled release system capable of interacting with the skin and promoting the drug control release directed to the superficial layers of the skin in order to avoid an undesired systemic absorption (CHANTASART et al., 2009; GELFUSO; CUNHA-FILHO; GRATIERI, 2016).

In this context, the nanostructured lipid carriers (NLCs) formed by solid and liquid lipids, which are the most modern generation of lipid nanoparticles, with more stability and higher drug loading capacity, emerges as a promising alternative (GELFUSO; CUNHA-FILHO; GRATIERI, 2016). In fact, NLCs have been used successfully in enhance the skin permeation of several drugs (CHAUDHARI, 2012), however, there is no studies involving the use of NLC with TML or natural extracts containing this compound. Nevertheless, the high reactivity of the lipid components (SHAHIDI; ZHONG, 2010; SILVA et al., 2016), could



compromise NLC over-all stability. In such context, compatibility studies between TML and lipid excipients are strongly demanded.

## 1.2 OBJECTIVES

Development of a nanostructured lipid carrier functionalized with the complex of TML and *Lippia origanoides* essential oil with cyclodextrin.

### 1.2.1 Specific objectives

- Develop and validate a chromatographic analytical method suitable for the determination of TML in drug delivery systems and to be use in skin permeation studies of TML topical formulations;
- Evaluate the drug-excipient compatibility between TML and NLC excipients using thermal and spectroscopic analyses following a mixture design;
- Select the best cyclodextrin type for complexation of TML and EO based on phase-solubility studies;
- Select the most suitable methodology for obtaining solid inclusion complexes considering several physicochemical properties, such as scanning electron microscopy, infrared spectroscopy, thermal analysis and dissolution rate;
- Prepare NLC of TML and EO in free and in inclusion complexed forms and analyze these systems by physicochemical assays, as well as liberation and permeation studies.

## 1.3 LITERATURE REVIEW

### 1.3.1 Skin disorders

In an adult, the skin has a total area between 18,000 cm<sup>2</sup> and 25,000 cm<sup>2</sup> and works as a barrier, avoiding the direct contact of the internal areas of the body with external agents. The skin is composed, fundamentally, of three layers, epidermis, dermis and hypodermis, been the first one the focus point for the topical drug delivery (LAUTERBACH; MÜLLER-GOYMANN, 2014; MONTENEGRO et al., 2016).

The epidermis is the most external skin layer. The cellular arrangement results in a compact and almost impermeable barrier, perfured only by the follicle polisebasea pores and the sweat glands. The stratum corneum, the outermost layer of the epidermis act to counteract

the loss of salts and water from the skin and the penetration of water soluble substances, having an important role in the protection of the body. In order to transpose the stratum corneum, the substances have to present a high liposolubility or have mechanisms that are capable to affect the cellular structure (LAUTERBACH; MÜLLER-GOYMANN, 2014).

The epidermis is a non-vascular area, so all the nutrition is provided by the more internal layers of the skin, particularly the dermis. Both the dermis and the hypodermis, because of the vascularization, are targets for drugs with systemic effects, but in topical drug delivery the contact with these layers have to be avoided, since, in this case, a systemic effect is considered toxic (GELFUSO; CUNHA-FILHO; GRATIERI, 2016).

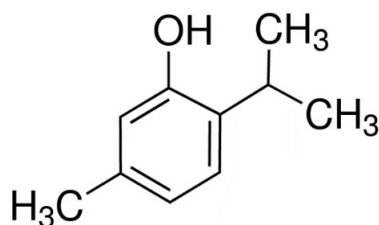
The skin is constantly in contact with external agents and that makes it vulnerable, making the skin disorders one of the most common pathological conditions in humans. Affecting about 70% of the population, these disorders can, slowly, reduce the quality of life of the individuals and, in critical cases, lead to incapacitation and death (BASRA; SHAHRUKH, 2005; BICKERS et al., 2006; FARAGE et al., 2012; HAY et al., 2014; HAY; FULLER, 2011).

Among the large variety of the skin disorders, some receive more attention, due to the severity of the symptoms and the possibility of endanger patients life, such as skin infections, melanomas and psoriasis (BASRA; SHAHRUKH, 2005; SWANSON; CANTY, 2013). However, another more common group of skin disorders with no imminent lethal risk but with clear impact on quality of life, such as acne, alopecia, irritations, degradations and many others, represent a font of shame and a psychological malaise which can evolve to more serious conditions affecting physical and mental health (GREEN, 2010).

In this sense, the research of new therapeutics strategies to treat and prevent some of those diseases are fundamental to improve the quality of life of people who suffer from these conditions. Presenting as one of the possible strategies are the molecule Thymol.

### 1.3.2 Thymol

Thymol (TML, Figure 1.1), 2-isopropyl-5-methylphenol, belongs to the cyclic phenolic monoterpene class and presents in its structure two isoprene units to form of a ring with a hydroxyl group bonded in this base structure forming a phenol (CHAPPELL, 1995; KUMAR; RAWAT, 2013; TAN et al., 2016). This molecule is a biological derivative of cymene and carvacrol and is founded in essential oil of several plants as the main component (ARCHANA; NAGESHWAR; SATISH, 2011).



**Figure 1.1** TML chemical structure (ANGELO et al., 2016)

The antiseptic, preservative and flavoring properties of TML and the essential oils containing TML explain the use of this component in food and personal care industry. In the food industry, TML are used to prevent microbiological contamination of the food inside the package during the storage process (ALTIERI et al., 2005; CASTILLO et al., 2014; KHAZAEI; ESMAILI; EMAM-DJOMEH, 2017). TML and its natural products are also used in personal care products as an antiseptic in oral hygiene products against halitosis and as flavoring agent in several products (JENTSCH et al., 2014; PRIESTLEY et al., 2003).

Several researches proved the therapeutical benefit of TML. Traditional medicinal use of TML are described to treat several problems, such as headaches, diarrhea, cough, worms, wounds and others (KAVOOSI; DADFAR; PURFARD, 2013; LEE et al., 2005; RIELLA et al., 2012). Antibacterial, antifungal, antiparasitic, antioxidant, anti-inflammatory, local anesthetic, cardiovascular modulator, anticancer and others effects are assigned to TML (BRAGA et al., 2006; DAMASCENO et al., 2011; HAESELER et al., 2002; KANG et al., 2016; MARCHESE et al., 2016; MENDES et al., 2010; SANTOS et al., 2011; TASDEMIR et al., 2006; YANISHLIEVA et al., 1999).

In this context, the use of TML in skin preparations seems to be very promising. Moreover, some studies reported the effect of TML as skin permeation enhancer (CHANTASART et al., 2009; MOHAMMADI-SAMANI et al., 2014). This means that TML could be used not just as an active product based on drug properties already mentioned but could as well act as an auxiliary product, responsible to enhancing the delivery of actives into the skin. This perspective expands the potential of TML to cosmetic field as well.

TML physicochemical properties such as low aqueous solubility, thermal and photo sensibility represents a barrier for its pharmaceutical exploitation (SHIMODA et al., 2006). In addition, for the topical administration of this product it will be necessary to develop drug delivery systems which could be able to control its permeation through the skin.

### 1.3.2.1 *Lippia origanoides*

The genus *Lippia sp* include more than 200 species of small trees, herbs and shrubs. One of these species are the *Lippia origanoides* (Figure 1.2), a shrub native from some countries of Central America and South America, in particular, from the Amazon region. In Brazil, the main habitat of these species is in Pará state, where the plant are known by the names of “Salva de Marajó” and “Alecrim d’Angola” (OLIVEIRA et al., 2006).



**Figure 1.2** *Lippia origanoides* (CALHAU, 2013)

The popular use of this plant is mainly for culinary and popular medicinal purposes. Studies pointed out that the infusion from the leaves and flowers of this plant were used in the treatment of stomachache, colic, indigestion, diarrhea, heartburn, nausea, flatus, vaginal discharge, menstrual complaints and fever. The use as antiseptic for mouth and throat is also reported (PASCUAL et al., 2001; TELES et al., 2014).

Most of the therapeutic effects described for this plant such as antimicrobial and cicatrizing are explained by the presence of TML in large amount of this natural product. However, there are evidences that the therapeutic potential of TML could be amplified due a synergic action among the fitocomplex composition of essential oil (OLIVEIRA et al., 2006; PASCUAL et al., 2001).

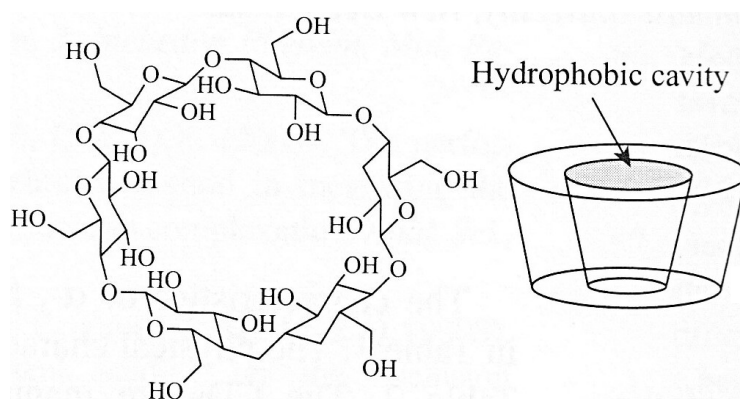
### 1.3.3 Cyclodextrin and inclusion complexes

CD were first synthesized in France by the years of 1890s as part of the work of the pharmacist and chemist Antoine Villiers. This scientist discovered that during the process of

degradation and reduction of carbohydrates, resultant from the fermentation of potato starch, a crystal with particular properties were produced, which due to its physical and chemical similarities with cellulose was named as “cellulosine” (CRINI, 2014).

About 20 years later, the Austrian chemist and bacteriologist Franz Schardinger characterized these structures as cyclic oligosaccharides present in two different forms, distinguished by solubility and by their reaction with iodine. He decided to rename these structures as dextrins — crystalline- $\alpha$ -dextrin and crystalline- $\beta$ -dextrin. For many years, CDs were call Schardinger dextrins, in recognition of the importance of his work in this field (CRINI, 2014; DEL VALLE, 2004).

Few years later, with the advent of crystallographic methods, the structure of the CDs was fully elucidated, and some different types of molecules were founded. It was discovered that CDs were formed by a grouping of D-glucopyranose unities arranged in a cyclic, hollow conical structure, and depending on the number of pyranose unities and the different chemical groups added to the molecule, different types of CD were formed (Table 1.1; Figure 1.3). Later these natural CDs were chemically modified by replacing their hydroxyl groups by others functional groups in order to modulate their properties. Today there are hundreds of varieties of modified CDs (MARQUES, 2010).



**Figure 1.3** Basic chemical structure of cyclodextrin and conformation model (Adapted from HIDETOSHI; MOTOYAMA; IRIE, 2011)

In the exterior portion of the CD are located the CH groups and the polar sugar hydroxyl groups that provide a very hydrophilic characteristic to that region. In the internal portion of CD, the opposite side of the structure are located, with rings of CH groups, a glucosidic ring with oxygens donors of hydrogen bonds in the extremity and the ether

oxygens, forming a region with high hydrophobic characteristics. Based on that, two of the main properties of the CDs are explained, the water solubility of these molecules, provide by the exterior hydrophilicity; and the tendency to forming a bond with molecules with hydrophobic groups in its internal portion, forming what are called inclusion complexes (MARQUES, 2010).

**Table 1.1** Types of cyclodextrins and characteristic (MARQUES, 2010).

<i>Cyclodextrin</i>	<i>Glucose unities</i>	<i>Molecular Weight (Da)</i>	<i>Cavity Dimentions (Å)</i>
$\alpha$ CD	6	972	4.5
$\beta$ CD	7	1135	7.0
$\gamma$ CD	8	1297	8.5
$\delta$ CD	9	1459	9.5

Inclusion complex are a form of chemical bond in which one molecule is encased on another molecule. The host molecule provides a protection of the included molecule, altering consequently its properties (DEL VALLE, 2004). The complexation occurs when, at the contact between the host (CD) and guest, the molecules are oriented in order to the most hydrophobic region of the guest molecule, interact with the most hydrophobic region of the CD, the internal region, and bonds are formed between the chemical groups of both structures and the result are a molecule surrounded by the CD (LOFTSSON; HREINSDÓTTIR; MÁSSON, 2005).

The complexation of guest molecules could alter substantially their properties. The exterior hydrophilic region of the CD is able to enhance the water solubility of the molecule and the structure of the CD produce a “shield effect”, protecting the molecule against external agents such as heat, light and others, enhancing considerably the molecule solubility and stability (ABARCA et al., 2015; SAMBASEVAM et al., 2013).

A relevant aspect to the CD inclusion complex formation and stability is regarding the size and tridimensional structure of guest and host (CRINI, 2014). The polarity of the guest is also important as, molecules with most hydrophobic regions on the structure are more propense to form stable inclusion complexes (MARQUES, 2010; SANTOS et al., 2015).

Moreover, studies show that many other forms of complexation are possible, such as the inclusion of more than one molecule in the same CD cavity and the non-included complexes, were the molecules interact with more external parts of the CDs. So even

molecule with unfavoured characteristics to form true inclusion complexes could form a different type of complex with CD by the interaction of functional groups (LOFTSSON; HREINSDÓTTIR; MÁSSON, 2005).

Not just the characteristic of the molecules plays an important role in the inclusion complex formation, but the preparation of these complexes can also affect the stability and strength of the bond between the molecules (SALÚSTIO et al., 2009). Specifically for the preparation of solid inclusion complexes, the methodologies which more successfully lead to inclusion complexes were freeze drying, spray drying, rotary evaporation and some more modern approaches such as supercritical fluid technology (RUDRANGI et al., 2015).

Freeze drying and spray drying are methods that involve the solubilization of the guest molecule and the CD in an aqueous solvent (mainly water) and then this solution are submitted to a drying process (SALÚSTIO et al., 2009). In the freeze drier, the solution is first frozen at very low temperature. In the equipment, a combination of a moderate heating and pressure forces the solvent sublimation until just the powder remains. The main advantages of freeze drying are the good yield of inclusion formation, the possibility of scale up the production and the use of moderate temperature making almost ideal for volatile guest molecules (SALÚSTIO et al., 2011; TAO et al., 2014).

In the spray drier, in turn, the solution is injected in the machine in liquid state. The equipment pulverizes the solution and immediately submit the resultant droplets to a high temperature treatment for a short period of time, which vaporize all the solvent. Because of the high temperature, the spray drying is recommended to be used with thermostable molecules. An advantage of this methodology is the high stability of the formed complexes. Indeed, the complexation efficiency of this method is usually high, since the instantaneously drying does not allow undo the complex previously obtained in the liquid phase (FERNANDES et al., 2009; FERNANDES; CANDIDO; OLIVEIRA, 2012).

In the rotary evaporation method, the guest molecule and the CD are solubilized in an organic solvent until the total solubilization of both. The solution is putted in a rotary evaporation equipment and a combination of high temperature and low pressure makes the solvent evaporates and leaves the sample in the powder form. Similarly as spray drying, the rotary evaporation deals with high temperature, possible not been suitable for volatile molecules, but the stability of the final product is reported to be stronger (FREITAS et al., 2012).

The supercritical fluid technology appeared as an alternative for the classic solvent methods. This technique is attractive because involves fewer steps and avoid the use of

organic solvents, mostly using CO<sub>2</sub> in supercritical state as the main solvent. This approach is an eco-friendly and efficient (timewise) method to produce interaction among materials in solid state (BANDI et al., 2004). Several different methods inside the supercritical fluid technology could be used to prepare an inclusion complex, depending of the characteristics of the guest and its solubility in supercritical CO<sub>2</sub> (DE SOUZA et al., 2017; HUANG et al., 2016; JUNIOR et al., 2017). Based in recent studies, the used of supercritical fluid is a very promising method to produce stable inclusion complexes with a broad spectra of molecules (RUDRANGI et al., 2015).

In order to evaluate the extension of inclusion complex formation in solid state as well as its properties, many assays could be used, such as scanning electronic microscopy (SEM), infrared spectroscopy (FTIR), thermal analysis (TA) and dissolution profile (DP).

SEM is a good option for assessing the morphological aspect of inclusion complex. The final form of the material can be related to the efficiency of the process. The CDs have a very characteristic form, in general amorphous, that changes with the complexation (PRALHAD; RAJENDRAKUMAR, 2004). In addition, the guests, normally in their crystalline form, are usually completely modified after inclusion complex. In general, the guests molecules are converted to an amorphous state (JUN et al., 2007).

Infrared spectroscopy is also a very common tool to evidence inclusion complex formation. In fact, the CD is capable of working as a “shield” for the guest, showing some alteration on the position and intensity of the characteristic bands of guest spectrum. Furthermore, the H bonds of CD with the guest molecule produce changes in the expected spectroscopic profile of the sample, providing additional evidences of complexation (CANNAVÀ et al., 2008; CRUPI et al., 2010; KAYACI; UYAR, 2011).

Thermal analyses are also useful for characterixation of inclusion complexes. Differential scanning calorimetry (DSC) provide consistent proofs of the influence of the CD on the guest molecules, based on its effect on the transition phase of the guest, such as melting or crystallization (MARQUES, 2010; MARQUES; HADGRAFT; KELLAWAY, 1990; PRALHAD; RAJENDRAKUMAR, 2004) The termogravimetry analysis (TGA), in turn, shows the thermal stability of the complexes produced in solid state (HUANG et al., 2016; JIAO; GOH; VALIYAVEETIL, 2001; KAYACI; UYAR, 2011).

As already pointed out, one of the most noticeable effect of the complexation of hydrophobic guest molecules is in its water solubility properties. Therefore, determination of intrinsic solubility as well as the evaluation of dissolution profile are very powerful tests to study the inclusion complex phenomenon (SÁ-BARRETO et al., 2013).



Thus, CDs and their inclusion complexes have been used in many areas with several applications in industry, medicine and research. In the pharmaceutical and cosmetical field, CDs are already used to optimize the characteristics of several drugs with repercussion on their bioavailability. More specifically for topical administration purposes, besides their capacity of modifying the physicochemical characteristics of the drugs, CDs have been able to modulate drug permeation through the skin, sometimes acting like a permeation enhancer and in other cases it can decrease the flux of drug through the skin layers (LOFTSSON; OLAFSSON, 1998).

#### **1.3.4 Topical drug delivery and nanostructured lipid carriers**

Strategies of drug delivery to epidermal layers are gaining prominence, since it presents many advantages over the systemic administration on numerous therapies, avoiding undesirable effects of the drugs which are distributed indiscriminately throughout the body. Besides that, the advantages of been less painful, non-invasive and comfortable make this kind of administration even more attractive (GELFUSO; CUNHA-FILHO; GRATIERI, 2016).

The main challenge for topical delivery on the epidermal layers is the surpass of the stratum corneum (SC). The SC is the most external and protective of the skin layers, due to its cell formation, composed of lipid and protein domains that limit the penetration of many molecules (MEHNERT; MÄDER, 2001).

Ideally, molecules for topical activity should present a low molecular weight (maximum of 500 Da); an intermediary partition coefficient making it relatively soluble in oil and water; and a highly potent action, capable of generating the desirable effect with a concentration as low as possible (preferable in a microgram per milliliter concentration range). The problem lays on the fact that even molecules with exceptional characteristics for SC penetration could not be suited for the topical administration, once a higher penetration can result in the drug arriving in more internal skin layers, such as the dermis, resulting in a systemic absorption, which causes undesirable effects. (GELFUSO; CUNHA-FILHO; GRATIERI, 2016)

Many strategies to overcome the SC barrier and also retaining the drug delivery in the skin layers have been developed in recent years, most of them involving the use of nanotechnology. A great variety of nanoparticles have been produced to control topical drug delivery. The main advantage of these systems is the potential of encapsulating the active,

protecting against external agents and delivering it on specific regions, depending on the composition of the particles and their compatibility with the active molecules (KIM; RUTKA; CHAN, 2010). For topical administration, one of the strategies that have been proved to have great results in the control of the permeation of actives in skin layers is the nanostructured lipid carriers (NLC) (CHAUDHARI, 2012).

NLC are a second generation of solid lipid nanoparticles formed by a solid lipid, in the exterior part of the particle and a liquid lipid, in the internal part (Figure 1.4). The liquid lipid is responsible to containing the active and because of its physical and chemical characteristics a numerous amount of molecules are capable to be encapsulated in the particle (TAMJIDI et al., 2013).



**Figure 1.4** Basic chemical structure of cyclodextrin and conformation model (MONTENEGRO et al., 2016)

NLC composition is formed by biodegradable and biocompatible material which permits that in contact with the skin, at physiological temperature, occurs a formation of an occlusive film increasing skin hydration and drug penetration. An important characteristic of NLC is the permeation rout of those system. NLC has a preference for a non-classic permeation rout, through the hair follicle (HF). Normally, the permeation thorough the skin layers are made by two main routes, the transcellular and intercellular, however the permeation through the hair follicle, previously neglected, has gained enormous notoriety recently. (MONTENEGRO et al., 2016). Indeed, the HF rout become a very efficient way to deliver molecules in skin layers and with the help of systems such as NLC making possible a sustain release of the active, avoiding toxic effects (GELFUSO; CUNHA-FILHO; GRATIERI, 2016).

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## **CHAPTER 2- DEVELOPMENT AND VALIDATION OF A SELECTIVE HPLC-UV METHOD FOR THYMOL DETERMINATION IN SKIN PERMEATION EXPERIMENTS**

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This part of the work aim to develop and validate a novel, simple and selective analytical HPLC-UV method for thymol determination, which would give support for the development and evaluation of dermatological and cosmetic products containing this natural substance.

### **2.3 MATERIAL AND METHODS**

#### **2.3.1 Chemicals and reagents**

Thymol ( $\geq 99.0\%$ ) was purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile of HPLC grade were purchased from Tedia Brazil (Rio de Janeiro, Brazil). Porcine skin was obtained from a local abattoir (Bonasa Alimentos, São Sebastião, Brazil). Scotch book tape no. 845 (3M, St Paul, EUA) and cyanoacrylate glue Loctite (Henkel, Dublin, Ireland) were used to perform the differential tape stripping technique. All assays were performed using ultrapure Milli-Q water (Millipore, IllkirchGraffenstaden, France).

#### **2.3.2 Samples preparation**

A stock solution of thymol (500  $\mu\text{g/mL}$ ) was prepared by dissolving 5 mg of the drug in 10 mL of methanol. Standard solutions were prepared by suitable dilution of the stock solution in methanol.

Stratum corneum (SC) and hair follicle (HF) were separated from the remaining porcine skin (RS) following the differential tape stripping technique, as previously described (MATOS et al., 2015a; PATZELT et al., 2008). After the differential tape stripping, each skin layer sample was placed in individual closed glass flasks with 5 mL of methanol under stirring (300 rpm, overnight). The solvent was then filtered through a 0.22  $\mu\text{m}$  membrane, producing skin extracts samples.

### 2.3.3 Chromatographic analysis

Thymol was quantified using a reversed-phase HPLC-UV method in a LC-20AD instrument (Shimadzu, Kyoto). UV detection was performed at 278 nm, oven temperature was 40 °C and the injection volume, 30 µL. Two columns were evaluated without the use of safe guards: (i) RP-C18 150 mm x 4.6 mm, 5 µm and (ii) RP-C18 300 mm x 3.9 mm, 10 µm. Different mobile phases and flow rates were tested to obtain the best peak resolution (Table 2.2).

### 2.3.4 Method Validation

The method was validated according to ICH guidelines (ICH, 2005), with respect to selectivity, robustness, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.

#### 2.3.4.1 *Selectivity*

Standard thymol solution (7.5 µg/mL) was evaluated in the absence and in the presence of each skin extract to ensure that endogenous compounds present in the skin would not interfere on the drug peak. All assays were performed in six independent sources of each matrix. Results were analysed considering peak area and retention time (RT).

#### 2.3.4.2 *Robustness*

Variations of  $\pm 5\%$  in three selected analytical parameters (percentage of water in the mobile phase, flow rate and oven temperature) were implemented according to a factorial design ( $2^3$ ), as presented in Table 2.1. All assays were performed in triplicate.

**Table 2.1** Factorial design for robustness evaluation of the developed method for quantification of thymol.

Trial	Mobile phase (acetonitrile:water v/v)	Oven temperature (°C)	Flow rate (mL/min)
1	34:66	38	1.425
2	34:66	38	1.575
3	36:64	38	1.425
4	36:64	38	1.575
5	36:64	42	1.425
6	36:64	42	1.575
7	34:66	42	1.425
8	34:66	42	1.575

A prediction equation was proposed for each response (i.e., peak area and RT) using the analysis of multiple regressions step-wise. The model was validated by the analysis of variance (ANOVA) with a significance level of 0.05. All statistical calculations were performed using the software Design-Expert version 9.

#### 2.3.4.3 Linearity

Three calibration curves were plotted in the range of 0.5 to 15 µg/mL of thymol and fitted using least squares linear regression. Angular coefficient significance and proportionality tests were evaluated based on the residual variance using student-t test ( $\alpha = 0.05$ ). Response factors were calculated considering the ratio between peak area and analyte concentration. The residues were calculated based on the difference between theoretical and experimental values calculated from the calibration curve (PINHO et al., 2016).

#### 2.3.4.4 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were estimated considering the ratio of background noise and analytical signal (ICH, 2005). Thymol LOD and LOQ were calculated based on the calibration curve and calculated according to Eqs. (1) and (2) (ICH, 2005):

$$\text{LOD} = 3.3 \sigma / S \quad (\text{Eq. 1})$$

$$\text{LOQ} = 10 \sigma / S \quad (\text{Eq. 2})$$

where  $\sigma$  = the standard deviation of the response, S = the slope of the calibration curve.

#### 2.3.4.5 Precision

Precision was evaluated in terms of repeatability and intermediate precision. Repeatability (intra-assay) was verified for three concentrations of thymol (0.5, 7.5 and 15.0 µg/mL) using three replicates of each. Intermediate precision was evaluated on two different days (inter-assay) using drug samples prepared by different analysts.

Results of precision were expressed as coefficient of variation (CV), calculated according to Eq (3):

$$CV = (\text{standard deviation of the mean} / \text{mean}) \times 100 \quad (\text{Eq. 3}).$$

#### 2.3.4.6 Accuracy

Accuracy was reported as percent recovery of known added amounts of thymol from skin layers (GRATIERI et al., 2012). The method consisted of spiking the skin samples with three different known amounts of drug in methanol solution (0.5; 7.5 and 15.0 µg/mL). After solvent evaporation, skin samples were cut into small pieces and soaked in 5 mL of methanol. Samples were left overnight under constant stirring (300 rpm) at ambient temperature and then were filtered and analyzed using the HPLC-UV method described above. Thymol recovery was determined by calculating the ratio of the amount extracted from the skin samples to the amount added, determined by direct injection of spiking solution in the absence of skin. The experiments were performed in triplicate. Results of accuracy were calculated according to Eq (4):

$$\text{Accuracy} = (\text{measured concentration} / \text{nominal concentration}) \times 100 \quad (\text{Eq. 4}).$$

## 2.4. RESULTS AND DISCUSSION

### 2.4.1 Optimization of chromatographic conditions

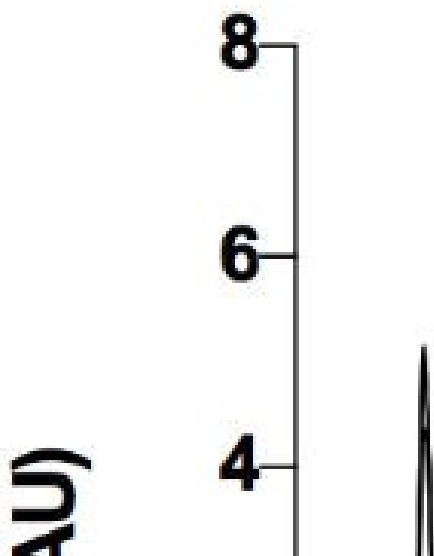
To evaluate the most adequate method for thymol quantification in skin samples, the thymol solution (7.5 µg/mL) and SC, HF, RS extracts were injected separately. The chromatograms and RT of each sample were then analyzed. As none of the previously reported methods for thymol determination (CHEN; CHEN, 2012; HAJIMEHDIPOOR et al., 2010; LI; YUAN; SU, 2006; SOLINAS; GESSA; DELITALA, 1981) could separate thymol peak from skin interferences, a series of modifications were performed. Data presented in Table 2.2 summarize the analytical conditions executed.

**Table 2.2** Variation in analytical conditions to develop HPLC-UV method for thymol quantification after skin permeation experiments.

Column	Trial	Mobile phase (v:v)	Flow rate (mL/min)	Thymol peak shape	Thymol RT (min)	Suitability
RP C <sub>18</sub> ; 150 × 4.6 mm; 5 μm	1	Methanol:water (70:30)	1.0	Tailing peak	6.471	Overleaping peaks
	2	Acetonitrile:methanol:water (25:50:25)	1.0	Tailing peak	3.720	Overleaping peaks
	3	Acetonitrile:methanol:water (15:50:35)	1.0	Tailing peak	6.326	Overleaping peaks
	4	Acetonitrile:methanol:water (10:50:40)	0.8	Tailing peak	11.251	Overleaping peaks
	5	Acetonitrile:water (60:40)	1.0	Tailing peak	4.805	Overleaping peaks
	6	Acetonitrile:water (30:70)	1.0	Tailing peak	6.254	Overleaping peaks
	7	Acetonitrile:water (25:75)	1.0	Suitable peak	8.286	Overleaping peaks
	8	Acetonitrile:water (25:75)	0.8	Suitable peak	10.384	Overleaping peaks
RP C <sub>18</sub> ; 300 × 3.9 mm; 10 μm	9	Acetonitrile:water (25:75)	1.0	Peak loss	–	–
	10	Acetonitrile:water (35:65)	1.0	Peak loss	–	–
	11	Acetonitrile:water (40:60)	1.0	Suitable peak	17.375	Overleaping peaks
	12	Acetonitrile:water (35:65)	1.5	Suitable peak	20.049	Suitable resolution
	13	Acetonitrile:water (35:65)	2.0	Suitable peak	15.912	Overleaping peaks

Initial tests (trials 1-6) were carried out with a RP-C18 (150 mm x 4.6 mm, 5 μm) column, evaluating methanol:water, acetonitrile:methanol, acetonitrile:water and acetonitrile:methanol:water as possible mobile phases (Table 2.2). In all of these conditions the analyte peak overlapped the skin interferences peaks. Additionally, thymol peak showed high asymmetry, with tailing factors reaching 3.738, which may be caused by solvation and viscosity effect of the mobile phase (HAUN; TEUTENBERG; SCHMIDT, 2012). This undesired interaction between the analyte and the mobile phase can be solved adjusting the force of solvent system (MCNAIR; POLITE, 2007). In this way, some modifications in the flow rate were performed (trials 7 and 8), but drug and interferences peaks still eluted at the same RT.

A chromatographic column with more length (RP C<sub>18</sub> : 300 × 3.9 mm; 10 μm) was tested in an attempt to improve peak resolution. Method trials number 9 and 10 resulted in peak loss, even following a 60 min running. This retention loss may have occurred due to the high content of water in mobile phase, which can fill column pores, turning them inaccessible to the analyte (WALTER; IRANETA; CAPPARELLA, 2005). Organic content in the mobile phase was thereby increased to 40% (v/v) (trial 11). However, drug RT was still in conflict with RT of matrices interferences. Hence, flow rate was adjusted in trials 12 and 13. Trial 12 moved thymol peak to a range out of skin interferences elution and was, therefore, selected for validation assays.



**Figure 2.2** Representative overlaid HPLC chromatograms of thymol solutions at different concentrations (0.5; 7.5 and 15.0  $\mu\text{g/mL}$ ). RP-C18 column (300mm x 3.9 mm, 10 $\mu\text{m}$ ), mobile phase of acetonitrile:water (35:65 v/v), flow rate of 1.5 mL/min, oven temperature at 40  $^{\circ}\text{C}$ , with injection volume of 30 $\mu\text{L}$  and detection at 278 nm.

Therefore, the selected method include the use a RP-C18 column (300 mm x 3.9 mm, 10  $\mu\text{m}$ ), with an isocratic mobile phase of acetonitrile:water (35:65 v/v) at a flow rate of 1.5 mL/min. The oven temperature was set at 40  $^{\circ}\text{C}$ , the injection volume was of 30  $\mu\text{L}$  and the detection occurred at 278 nm. Thymol RT was 20.5 min, with a running time of 27 min (Figure 2.2).

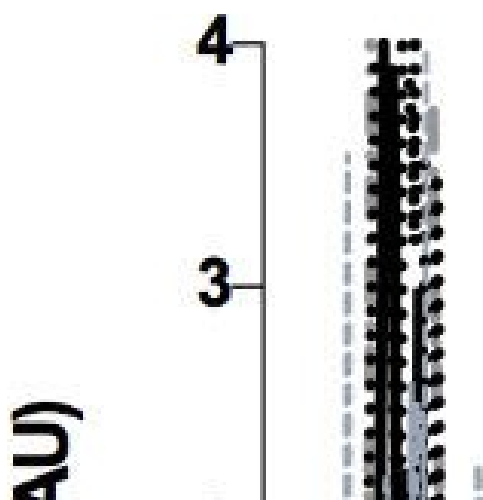
The efficiency of the method is expressed by the number of theoretical plates (N), which assumed the value of 9325 and is in agreement with FDA's established parameter of  $N > 2000$  (FDA, 1994). The symmetry of the peak is established by the tailing factor (T), which was of 1.035, consistent with the limit of  $T < 1.5$ , defined by the USP (BONFILIO et al., 2012).

## 2.4.2 Validation

### 2.4.2.1 Selectivity

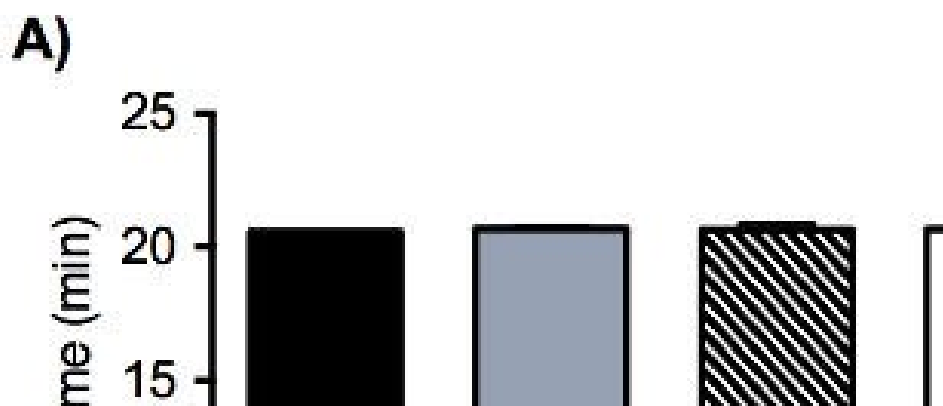
It is observed in Figure 2.3 that most contaminants from the skin fractions eluted over the first 10 min of running, with some peaks eluting until 18 min. For the SC fraction, an additional contamination peak was noted at 24 min. Thymol peak eluted after 20 min, free of any interference from solvent or skin components.





**Figure 2.3** Representative overlaid HPLC chromatograms of SC = stratum corneum; HF = hair follicle; RS = remaining skin and TML = thymol . RP-C18 column (300mm x 3.9 mm, 10 $\mu$ m), mobile phase of acetonitrile:water (35:65 v/v), flow rate of 1.5 mL/min, oven temperature at 40 °C, with injection volume of 30 $\mu$ L and detection at 278 nm.

Figure 2.4 shows skin matrix interferents did not alter drug RT. Likewise, thymol peak areas were not changed in the presence of the biological matrix, confirming the method selectivity.



**Figure 2.4** Selectivity of the method in quantification of thymol (7.5  $\mu$ g/mL) alone and added to skin layer extracts. Analysis in terms of (A) retention times and (B) peak areas. All assays performed with six independent sources of each sample. SC = stratum corneum; HF = hair follicle; RS = remain skin.

### 2.4.2.2 Robustness

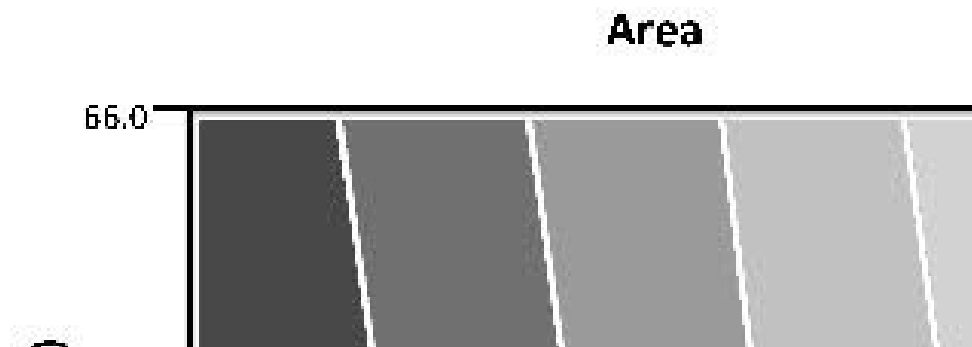
A predictive model was constructed to elucidate how each response (thymol peak area and RT) behaves according to variations in analytical parameters and their mutual interactions (Eqs. (5) and (6)).

$$\text{Area} = + 2.3 \times 10^5 - 447.8 \text{ WC} + 121.5 \text{ T} - 65,452.2 \text{ FR} \quad (\text{Eq. 5})$$

$$\text{RT (min)} = - 238.7 + 4.5 \text{ WC} - 0.3 \text{ T} - 98.4 \text{ FR} - 1.7 \text{ WCT} \quad (\text{Eq. 6}),$$

where WC is the water content (% v/v); T is the oven temperature (°C); and FR is flow rate (mL/min).

The proposed predictive model for peak area had a high statistical significance ( $p < 0.0001$ ) with  $R^2$  of 0.9864, which allowed for predictions within the design space with a high degree of accuracy. No significant interaction was identified among the three studied factors. Considering the variation margins studied, only the flow rate caused important changes in the peak areas of thymol. Lower flow rates produced higher molecular diffusion time in the column, resulting in broaden peaks (KAZAKEVICH, 2007). Indeed, the response surface graph presented in Figure 2.5 clearly shows the increase in flow rate, even within narrow margins, reduced the values of thymol peak area (negative coefficient of the equation). Variations in temperature and water percentage in the mobile phase within studied ranges did not generate significant changes in peak areas of the chromatograms assigned to the analyte.



**Figure 2.5** Response surfaces for thymol peak area and retention time in robustness assay.

In case of RT, the proposed predictive model ( $p < 0.0001$ ) also presented a high predictive ability ( $R^2 = 0.9894$ ). The model is composed by the three factors, also including the interaction between water content and flow rate. For this response, all terms of equation were statistically significant, and variations in flow rate, once again, produced the most

pronounced changes in thymol RT. This result was expected, since the peak position peak is strongly influenced by the flow rate (MCNAIR; POLITE, 2007).

Variations of the column temperature also had an impact on the drug RT due to the viscosity changes on the mobile phase; however, such impact was considerably less extreme compared to that produced by flow rate variations (equation coefficients  $\propto$  300-fold lower).

The polarity of the mobile phase, as might be expected, also influenced the drug RT. Small changes in water percentage in the mobile phase increased polarity and shifted the analyte peak (MCNAIR; POLITE, 2007).

Combination of deliberate variations in water content or in the flow rate changed the RT (Figure 2.5). The combination of these factors was especially relevant for such response.

In general, the developed analytical method presented good sensitivity. Hence, oscillations found in area response due the studied factors did not bring great impact on the analytical method sensitivity. Regarding RT, analyzed variations in chromatographic conditions produced changes not only for the analyte peak but also for the interferences. As interferences and analyte peaks shifts may not always be proportional, in some conditions method selectivity may be compromised. Thus, the necessity of a strict control over the flow rate is evident. Equipment preventive maintenance should be sufficient to ensure accurate operation of the pumps, avoiding oscillations on flow rate and mobile phase mixture (MCNAIR; POLITE, 2007).

#### 2.4.2.3 Linearity

Linear regression of the calibration curve obtained the following equation:  $y = 13,395x - 502.1$ . The high numerical value of slope (13,395) indicates a good methodological sensitivity. This result was expected based on the wide concentration range proposed (0.5–15  $\mu\text{g/mL}$ ). Student's t-test showed the slope was different from zero, with a confidence interval from 13.1 to 13.7.

The independent term ( $-502.1$ ) can be attributed to the systematic error of the method, as demonstrated by the proportionality test that shows a confidence interval between  $-2,894$  and  $1890$ .

The results of least squares linear regression analysis showed that the correlation coefficients of all the standard curves were  $\geq 0.999$ , which complies with all regulatory requirements for this parameter (SHABIR, 2004).

Calculation of residues showed a random data distribution with equivalent numbers of

positive and negative residues, with no tendency. The response factors calculated from each of the curve points were very similar among each other and close to the numerical value of the slope. Coefficient of variation of the response factors was around 2.5%, within the acceptable limit of 5%, ratifying the linearity of the method (MATOS et al., 2015b; SHABIR, 2004).

#### 2.4.2.4 Limit of detection and limit of quantification

The obtained LOD and LOQ values were, respectively 0.05 µg/mL and 0.14 µg/mL. Such values may be sufficient for determination of drug permeation kinetics profile into the skin from initial sampling points.

#### 2.4.2.5 Precision

The proposed method was quite resistant to the variables proposed, with coefficient of variation values lower than 2% (Table 2.3). This data is below the limits of 5% for bioanalytical methods by a large margin, and also meets the recommended limits for methods employed in assays of raw materials and dosage forms establishing a maximum coefficient of variation of 2%.

**Table 2.3** Results of precision tests for determination of thymol in standard solutions.

Thymol TC <sup>a</sup> (µg/mL)	Repeatability		Intermediate precision	
	Thymol MC <sup>a</sup> (µg/mL)	CV <sup>a</sup> (%)	Thymol MC <sup>a</sup> (µg/mL)	CV <sup>a</sup> (%)
0.50	0.49	0.73	0.52	1.35
7.50	7.44	0.38	7.56	0.83
15.00	15.49	0.32	15.40	0.99

<sup>a</sup> TC = theoretical concentration; MC = measured concentration; CV = coefficient of variation.

#### 2.4.2.6 Accuracy

Table 2.4 summarizes the accuracy found for recovered thymol, with values higher than 90% for all drug concentrations and biological matrices evaluated. Thymol recovery achieved in HF matrix was close to 100%. Such results eliminate the need of using a correction factor during the permeation experiments, as according to the literature drug recovery rates from skin above 70% can be acceptable (CAMPOS; PRAÇA; BENTLEY, 2015; DE PAULA; MARTINS; BENTLEY, 2008; VÁVROVÁ et al., 2007).

**Table 2.4** Results of precision tests for determination of thymol recovered from skin layers.

Thymol TC <sup>a</sup> (µg/mL)	Stratum corneum			Hair follicle			Remaining skin		
	Thymol MC <sup>a</sup> (µg/mL)	CV <sup>a</sup> (%)	Recovery (%)	Thymol MC <sup>a</sup> (µg/mL)	CV <sup>a</sup> (%)	Recovery (%)	Thymol MC <sup>a</sup> (µg/mL)	CV <sup>a</sup> (%)	Recovery (%)
0.5	0.46	5.17	92.00	0.50	8.37	100.00	0.49	8.34	98.00
7.5	6.95	8.13	92.67	7.43	3.85	99.07	7.32	3.16	97.60
15.0	14.42	3.37	96.13	15.04	2.73	100.27	14.82	4.56	98.80

<sup>a</sup> TC = theoretical concentration; MC = measured concentration; CV = coefficient of variation.

Coefficients of variation found for this validation parameter were below 10%, meeting the set limits by international health authorities, which is of 15% in case of bioanalytical methods (BANSAL; DESTEFANO, 2007; SHAH et al., 2000).

## 2.5 CONCLUSION

Biological matrices are complex and present two major analytical challenges: the drug recovery from the matrix and drug separation from interferences during the analytical procedure. The diverse nature of contaminants found in the different skin layers is an additional factor of difficulty. The developed analytical method circumvented all these challenges. It was selective for thymol and presented recovery rates higher than 90% using a simple HPLC-UV apparatus. The validation showed the method might be useful for *in vitro* skin permeation studies of topical dermatological or cosmetic products containing thymol.

## 2.6 ACKNOWLEDGMENTS

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## **CHAPTER 3- USE OF MIXTURE DESIGN IN DRUG-EXCIPIENT COMPATIBILITY DETERMINATIONS: THYMOL NANOPARTICLES CASE STUDY**

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This part of the work describes an experimental mixture design associated with thermal and spectroscopic analyzes as a tool to define each formulation component contribution on TML stability. The aim is to determine possible interactions between components and to evaluate risks of associated excipients. Despite guiding formulation development, this paper suggests a new experimental approach for compatibility studies.

### **3.4 MATERIAL AND METHODS**

#### **3.4.1 Materials**

TML (Lot SZBF0370V, purity  $\geq 99\%$ ) and sodium taurodeoxycholate (TAU, purity  $\geq 95\%$ ) were purchased from Sigma-Aldrich (Steinheim, Germany). Stearic acid (SA) was purchased from Dinâmica Química Contemporânea Ltda (São Paulo, Brazil). Soybean Lecithin (LC) was purchased from Lipoid (Ludwigshafen, Germany). Polysorbate 80 (P80) was purchased from Merck (Darmstadt, Germany).

#### **3.4.2 Experimental mixture design**

A drug-excipient compatibility study with TML and selected excipients commonly used to prepare nanostructured carriers was conducted following a simplex centroid mixture design with three components, without constraints (ERIKSSON; JOHANSSON; WIKSTROM, 1998). In the design A, fatty acid SA and phospholipid LC were mixed with the anionic surfactant TAU. In the design B, TAU was replaced by the nonionic surfactant P80. Mixtures were prepared in a mortar containing 50% (w/w) of TML and 50% (w/w) of other components according to the mixture design (Table 3.1). Experiments were performed randomly. The responses obtained from thermal analysis (TML melting peak variation, TML evaporation peak variation and excipient decomposition peak variation) and spectroscopy assays (sample stability based on correlation coefficient of spectra) were analyzed using the software Design Expert 8.0 (Stat-Ease, USA). The possible models were analyzed using

ANOVA one-way. The best fitting model was selected for each response based on *p-value* and a predictive equation containing only significant terms was built from stepwise multiple regression analysis.

**Table 3.1** Mixtures composition studied according to the simplex centroid mixture design. TML: thymol; LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

Mixture	TML (%, w/w)	SA (%, w/w)	LC (%, w/w)	TAU ( <i>design A</i> ) or P80 ( <i>design B</i> ) (%, w/w)
1	50	50.0	-	-
2	50	-	50.0	-
3	50	-	-	50.0
4	50	25.0	25.0	-
5	50	25.0	-	25.0
6	50	-	25.0	25.0
7	50	16.7	16.7	16.7

### 3.4.3 Thermal analysis

Differential thermal analysis (DTA) and thermogravimetric analyses (TGA) were performed simultaneously using a Shimadzu® DTG-60 series under nitrogen controlled atmosphere with flow of 50 mL·min<sup>-1</sup>. Samples of approximately 3 mg were analysed in a platinum pan in a heating range of 25 °C - 450 °C using a heating rate of 10 °C min<sup>-1</sup>. TML melting peak was calculated from DTA data and  $T_{peak}$  for TML evaporation and excipient decomposition were obtained from the first derivative of mass loss curves of TGA. Thermal measurements were determined using the TA-60 Shimadzu® software.

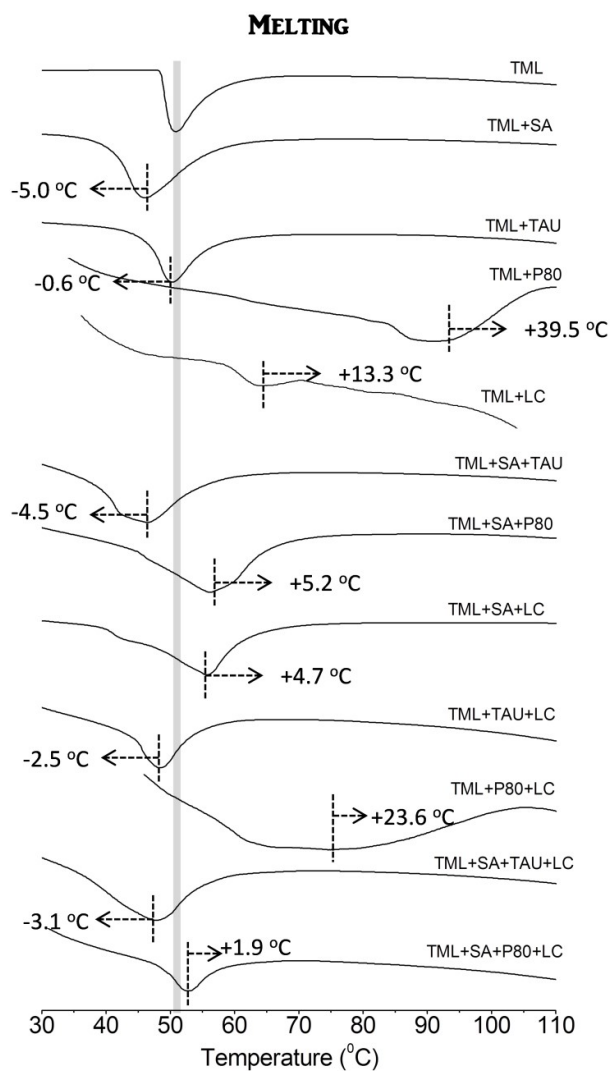
### 3.4.4 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analyses were performed using a Varian 640-IR FTIR spectrometer (Varian Inc., Brazil). The spectra were recorded between 650 cm<sup>-1</sup> and 4000 cm<sup>-1</sup> using an ATR imaging accessory. Individual compounds and mixtures of TML and excipients were evaluated before and after a thermal stressing (heating samples until 80 °C). The resulting spectra were analyzed considering the correlation coefficient (*r*) between samples based on

the bands corresponding to the functional groups of the drug and the excipients using the Essential FTIR software (Operant LLC, USA).

### 3.5 RESULTS AND DISCUSSION

#### 3.5.1 Thermal analysis



**Figure 3.1.** DTA curves of thymol (TML) and its mixtures according to the mixture designs. TML melting peak is shaded and the shifts on melting temperature are indicated in each mixture. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

In simultaneous DTA-TGA analysis the melting of TML occurred at 50.9 °C with an associated endotherm of 923 J·g<sup>-1</sup>, followed by evaporation of the drug in the range of 67 °C – 144 °C ( $T_{\text{peak}} = 139.9$  °C). In the mixtures with selected excipients, the correspondent thermal events of each component plus the decomposition of excipients, which occurs at temperatures above 200 °C, could be observed. Both TML characteristic thermal events, as well as excipients decomposition were used as thermal control parameters to study the compatibility among compounds.

There is a noticeable thermal interaction in almost all studied systems, with drug melting peak shifts from -5 °C to +39.5 °C regarding the expected temperature of 50.9 °C. In contrast, in TML+TAU binary mixture the expected melting peak temperature was maintained (Figure 3.1).

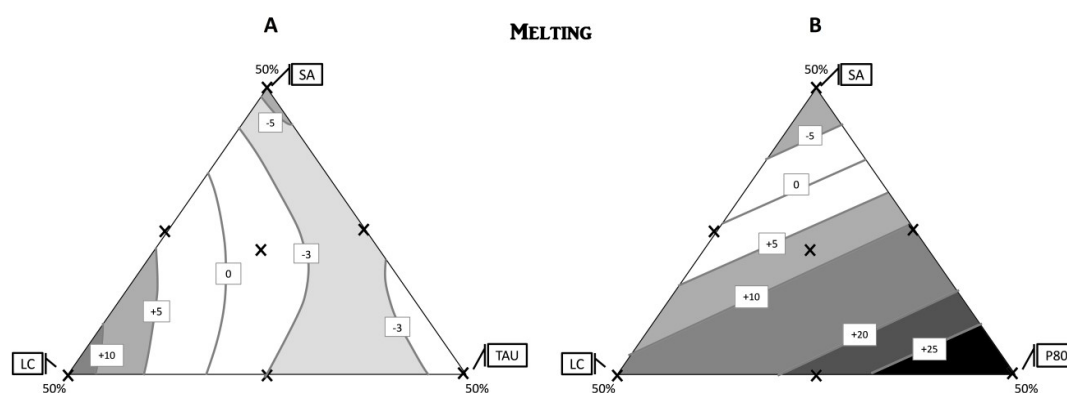
In mixtures containing SA (Figure 3.1), an overlap of TML and fatty acid melting was observed to a temperature lower than expected, which suggests the formation of an eutectic mixture (KANG; JUN; MANI, 2001; ZHANG et al., 2014). Both LC and P80 caused a pronounced displacement of TML melting peak to higher temperatures. In the case of LC, a marked reduction in the expected enthalpy was further observed, probably following drug solubilization. Noteworthy, TML melting was increased in more than 20°C in the ternary system containing LC and P80 (Figure 3.1).

**Table 3.2.** Fitting model, p value, F value, predictive equation and regression coefficient ( $R^2$ ) for each response of the mixtures designs (A and B). LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate; TML: thymol.

Response		Model	P value	F value	Predictive equation	$R^2$
Melting	A	Quadratic	0.0037	58.34	$\Delta T_{\text{peak}} (^{\circ}\text{C}) = -0.11\text{SA}\% + 0.27\text{LC}\% - 0.03\text{TAU}\% - 0.02\text{LC}\%:\text{TAU}\%$	0.96
	B	Linear	0.0435	7.59	$\Delta T_{\text{peak}} (^{\circ}\text{C}) = -0.20\text{SA}\% + 0.24\text{LC}\% + 0.67\text{P80}\%$	0.84
Evaporation	A	Linear	0.0428	7.66	$\Delta T_{\text{peak}} (^{\circ}\text{C}) = +0.08\text{SA}\% + 0.17\text{LC}\% - 0.29\text{TAU}\%$	0.79
	B	Quadratic	0.0283	34.53	$\Delta T_{\text{peak}} (^{\circ}\text{C}) = +0.08\text{SA}\% + 0.24\text{LC}\% - 0.04\text{P80}\% - 0.01\text{SA}\%:\text{LC}\% - 0.005\text{LC}\%:\text{P80}\%$	0.99
Excipient decomposition	A	Quadratic	0.0206	17.71	$\Delta T_{\text{peak}} (^{\circ}\text{C}) = -0.32\text{SA}\% - 0.007\text{LC}\% - 0.06\text{TAU}\% - 0.03\text{SA}\%:\text{TAU}\%$	0.95
	B	Linear	0.0451	7.42	$\Delta T_{\text{peak}} (^{\circ}\text{C}) = -0.35\text{SA}\% - 0.007\text{LC}\% - 0.15\text{P80}\%$	0.79
Stability	A	Quadratic	0.0023	427.71	$r = +0.020\text{SA}\% + 0.020\text{LC}\% + 0.009\text{TAU}\% + 0.0002\text{SA}\%:\text{TAU}\% + 0.0004\text{LC}\%:\text{TAU}\%$	0.99
	B	Quadratic	0.0399	10.97	$r = +0.02\text{SA}\% + 0.02\text{LC}\% + 0.02\text{P80}\% - 0.0009\text{SA}\%:\text{P80}\%$	0.92

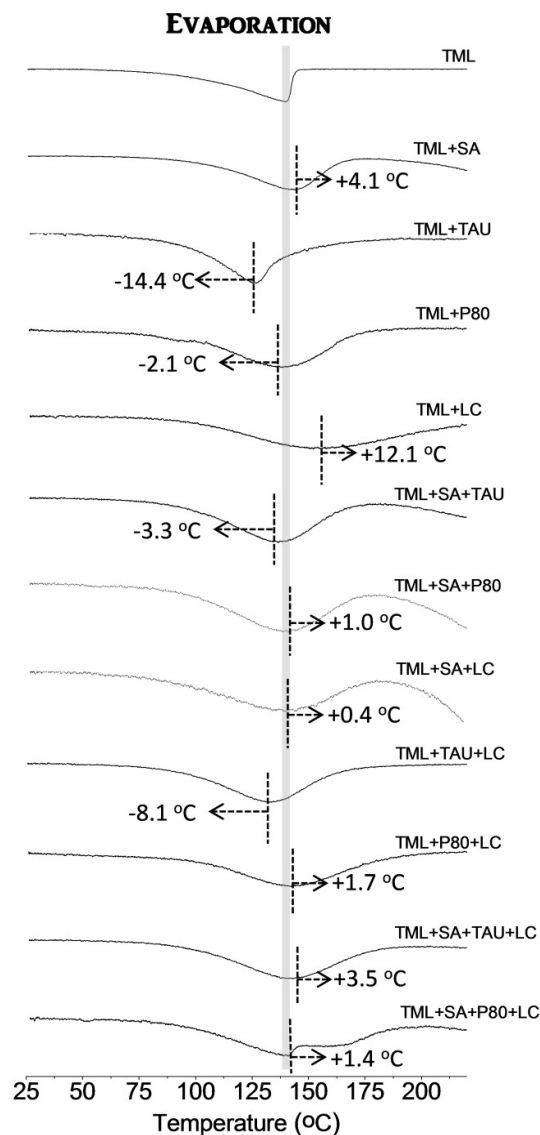
TAU was the only excipient that did not change the melting peak of TML (Figure 3.2A); however, a significant interaction of this surfactant with LC was observed, resulting in an retardation of TML melting event (negative term in predictive equation of Table 3.2).

According to surface response for TML melting peak variation (Figure 3.2), P80 is the component that has more influence on this response, strongly shifting the melting peak to higher temperatures (darker regions of Figure 2B). In the predictive equation for design B, the coefficient for P80 is much higher than those related to the other two components (Table 3.2). P80 has multiple proton acceptors in its molecular structure that could form hydrogen bond with the proton donor of TML (KUMBHAR; POKHARKAR, 2013). The increase of intermolecular forces hinders drug phase transition, enhancing TML melting peak (WESTWELL et al., 1995).



**Figure 3.2.** Response surface for thymol (TML) melting according to mixture designs A and B. Dark areas show regions with higher thermal interaction. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

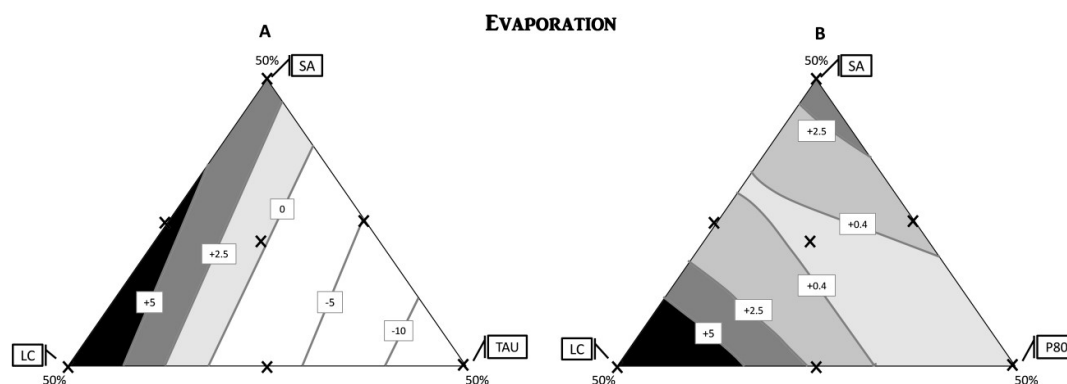
TML evaporation is a critical property for the preparation of NLC, which requires heating. This can cause loss of the drug at temperatures above 50 °C. In some of the studied systems, the presence of excipients in composition stabilized TML by increasing its evaporation temperature (Figure 3.3). This effect was more pronounced in samples containing LC. The TML+LC mixture, for example, showed an increase in evaporation peak of approximately 12 °C. The solvation and subsequent solubilization of TML in the phospholipid stabilized the mixture, delaying drug evaporation. However, the opposite effect was observed in some mixtures, as such containing TAU, where an anticipation of the drug evaporation was detected (Figure 3.3).



**Figure 3.3.** First derivative from mass loss TG curves of thymol (TML) and its mixtures according to the mixture designs.  $T_{peak}$  for TML evaporation is shaded and the shifts on  $T_{peak}$  are indicated in each mixture. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

Dark regions in Figure 3.4 indicate compositions with greater TML physical stability, representing more adequate compositions for the preparation of TML-loaded NLC. Predictive equations (Table 3.2) highlight the role of SA and LC in promoting an increase in TML evaporation temperature (positive sign in the equations). The surfactants (TAU and P80), on the other hand, act by reducing the temperature at which this phenomenon occurs (negative sign in the equations). TAU is the most relevant factor for this answer, since its coefficient

shows the highest numerical value in equation (Table 3.2). Also, the negative interaction between SA and LC and, between LC and P80, represents an antagonistic action of these components, reducing TML physical stability (Table 3.2).

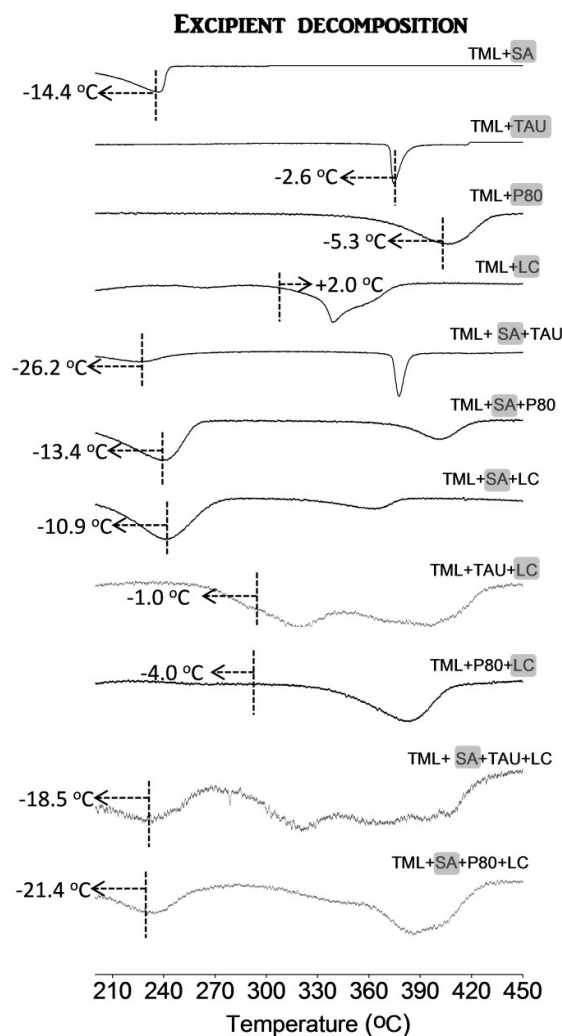


**Figure 3.4.** Response surface for thymol (TML) evaporation according to mixture designs A and B. Dark areas show regions with TML higher physical stability. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

Systems decomposition degree measured by TGA is an important parameter to access sample stability. The use of the first derivative of mass loss curves provided by TGA allows distinguishing the decomposition events of each component, even in complex mixtures (SILVA et al., 2016a). Figure 3.5 shows the  $T_{peak}$  related to these events and highlights the shifts on this value comparing to the behavior observed for each isolated material. The shifts were calculated based on the component that first underwent decomposition in the mixture.

Almost all mixtures showed anticipation of excipient decomposition, except for TML+LC sample (Figure 3.5). Complex samples containing three or four components revealed more marked reduction in stability, which reinforces the need to study the compatibility of systems considering the combination of multiples components. Some of those mixtures, e.g. TML+SA+TAU and TML+SA+LC+P80, anticipated the expected decomposition temperature in more than 20°C (Figure 3.5).

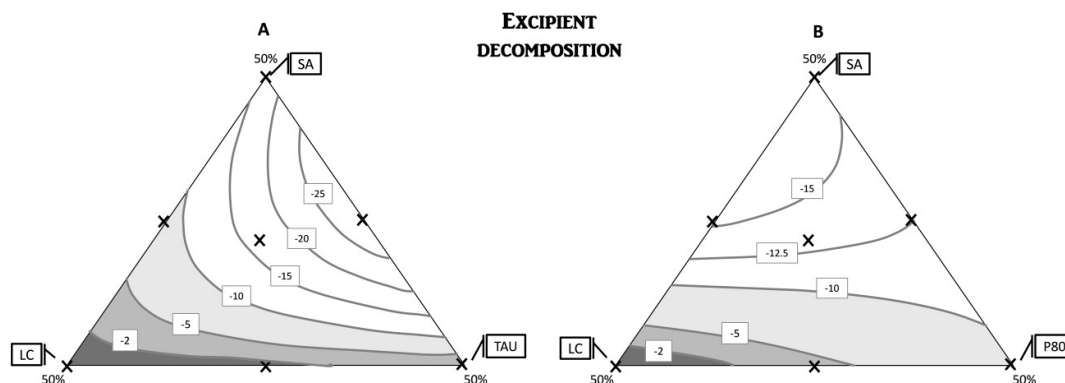




**Figure 3.5.** First derivative from mass loss TGA curves of thymol (TML) and its mixtures according to the mixture designs. The shifts on  $T_{peak}$  for TML excipient decomposition shaded are indicated in each mixture. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

In this case, the region of response surface that best preserves the sample involves higher concentrations of LC (Figure 3.6). The inert relation of LC with TML could be related to the solubilization of the drug in this lipid, as previously observed. According to the predictive equation (Table 3.2), LC showed the lowest coefficient value for this answer. The opposite behavior was observed for SA, which had a negative signal and the largest coefficient value, indicating that this component makes the sample less stable at high temperatures (Table 3.2). A negative interaction was observed between SA and TAU, as well

as between SA and LC, which showed the association of these components has an antagonistic effect on system stability (Table 3.2).



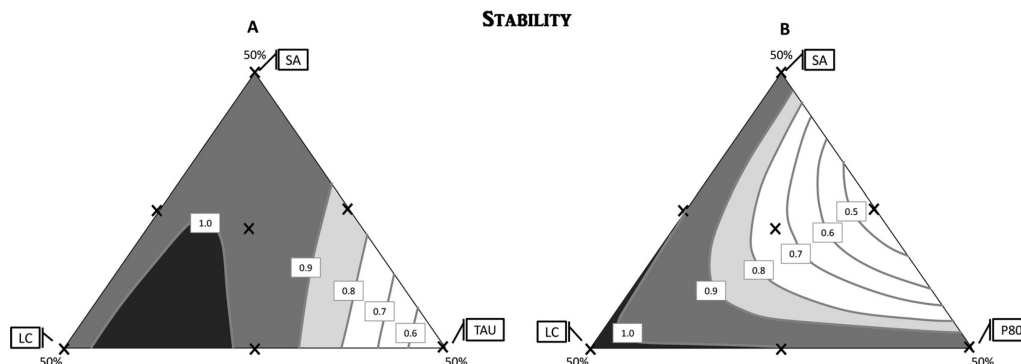
**Figure 3.6.** Response surface for thymol (TML) excipient decomposition according to mixture designs A and B. Dark areas show regions with excipient higher stability. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

### 3.5.2. Spectroscopic studies

Each formulation isolated compound and in blends was analyzed by FTIR before and after thermal stress. In order to obtain a numerical response representative from FTIR data for the mixture design evaluation, a correlation coefficient ( $r$ ) was calculated based on the similarity between the spectra before and after thermal stress. Values of  $r > 0.9$  indicated a good level of structural similarity between the samples (MECOZZI; PIETROLETTI; MONAKHOVA, 2016). The analysis of individual compounds before and after thermal stress showed  $r$  within this limit, indicating the treatment used preserves integrally the spectroscopic profile of the components alone. In the mixtures, the values for  $r$  ranged between 0.9992 and 0.8119, suggesting incompatibilities in some samples.

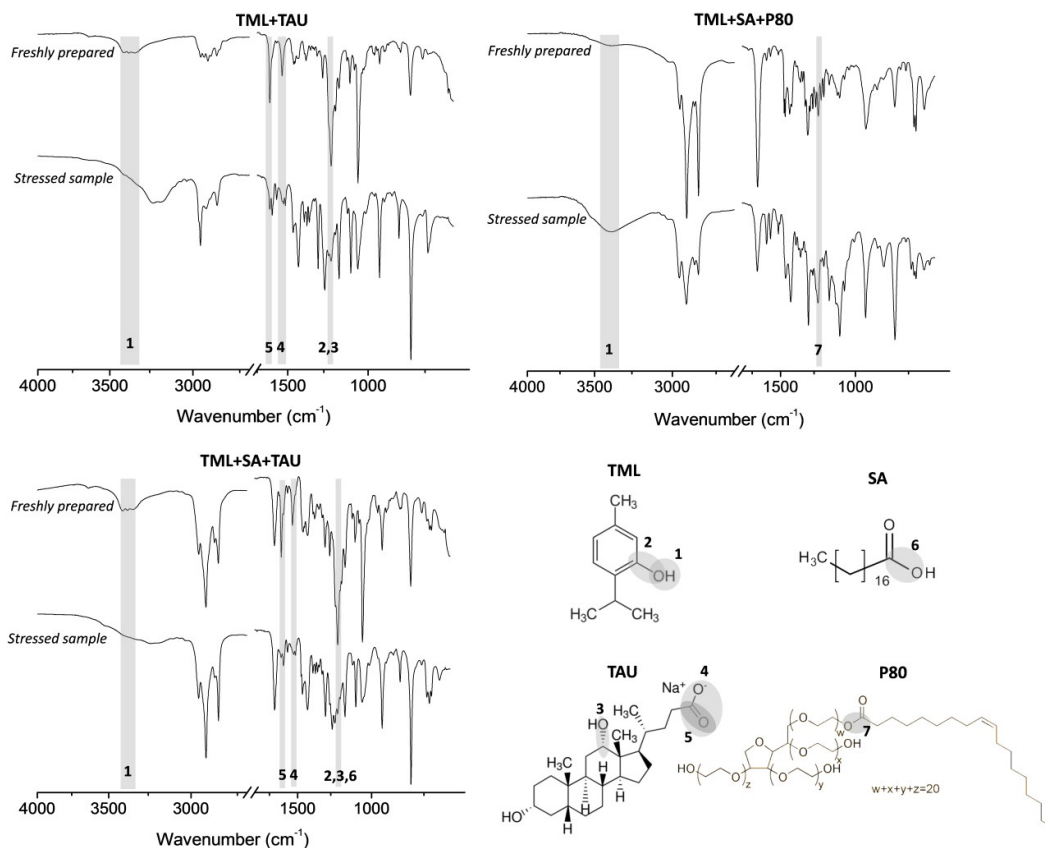
Similarly to thermal analysis results, better stability was achieved with higher proportions of LC (darker areas in Figure 3.7). In turn, TAU exerted a negative effect on the system stability (blank areas in Figure 3.7A), showing the smallest coefficient on predictive equation. In addition, the excipient interacted with the two lipid components (SA and LC) compromising the stability of the sample (Table 3.2). The substitution of TAU by P80 in design B showed that compounds either alone or in certain proportions produce stable systems; however, a negative interaction between SA and P80 is observed, which decreases the

stability of the sample in higher amounts of those components (Table 3.2, blank area in Figure 3.7B).



**Figure 3.7.** Response surface for thymol (TML) excipient decomposition according to mixture designs A and B. Dark areas show regions with higher formulation stability. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate.

In total, three of the analyzed mixture presented  $r$  value below the acceptable limit of similarity (Figure 3.8). After thermal stress, the binary mixture of TML and TAU and the ternary mixture of TML, SA and TAU, showed a displacement of the peaks related to the -OH stretch and C-O stretch (at  $3360\text{ cm}^{-1}$  and  $1205\text{ cm}^{-1}$ , respectively). The ternary mixture of TML, SA and P80 also presented some differentiation in peaks relating to the -OH stretch and the C-O stretches (at  $3430\text{ cm}^{-1}$  and  $1222\text{ cm}^{-1}$ , respectively). Those displacements could be a result of hydrogen bond between the hydroxyl group, which acts as a hydrogen bond donor and the carbonyl group, which acts as a hydrogen bond acceptor (KUMBHAR; POKHARKAR, 2013; LU et al., 2011). However, the sum of the modification in the spectra together with the changes in position and relative intensity of the peaks, especially in the sample TML+SA+TAU, suggests more than a physical interaction, indicating sample decomposition.



**Figure 3.8.** Chemical structures of the compounds and FTIR spectra of selected mixtures before and after thermal treatment. Changes in the spectrum are shaded and numbered according to the functional groups involved. LC: soybean lecithin; P80: polysorbate 80; SA: stearic acid; TAU: sodium taurodeoxycholate, TML: thymol.

### 3.6 CONCLUSION

Even though TAU did not interact with TML when in solid state, after drug melting, physical and chemical instability could be observed, drug evaporation and decomposition were anticipated, and significant changes in infrared spectra were observed. SA, in turn, did not compromise TML stability alone, but demonstrated negative interaction when in combination with other formulation components. LC solubilized TML, elevating the evaporation temperature and benefiting system chemical stability when presented in higher proportions. P80 exerted great influence on TML thermal events; still without compromising physical or chemical stability. This surfactant therefore must be a good alternative to the development of nanostructured lipid systems containing TML.

Although the quantitative delineation of formulation components will be defined by production and performance parameters, the safest formulation to enhance the stability of nanostructured lipid carriers containing TML should comprise P80 as a surfactant and higher amounts of LEC, while keeping SA concentration in low levels. The use of mixture design in drug-excipient compatibility studies was able to provide more complete information on drug stability profile and guide the development of stable formulations.

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## CHAPTER 5 – FINAL CONSIDERATIONS

A HPLC-UV analytical method was developed for TML determination. The method was selective against skin contaminants and presented recovery rates higher than 90% from skin layers. The validation showed the method might be suitable for *in vitro* permeation studies of topical products containing TML.

TML-excipient compatibility studies using a mixture design protocol were useful to provide more complete information on drug stability profile and guide the development of NLC stable formulations, preventing the use of incompatible components such as TAU, which presented a synergic effect with the other components of NLC, reducing the TML stability.

The process of complexation with CDs was able to manipulate successfully the physicochemical characteristics of TML and EO, originating a more stable product, therefore enhancing the amplitude of its applications.

The CD type and the method of production are decisive factors for a satisfactory formation of inclusion complexes with TML and its natural substrate (EO). Indeed, a better complexation efficiency was achieved using HP $\beta$ CD, while the freeze-drying method proved to be more effective in produce true inclusion complexes in solid state.

The encapsulation with HP $\beta$ CD was able to overcome the volatility and low aqueous solubility of TML and the insertion of these complexes into NLC was able to control the release and the permeation of the active component. Moreover, the inclusion complexes altered the permeation dynamics of TML in the skin, keeping its release circumscribed to the skin initial layers, vectoring the topical treatment.

Thus, the NLC systems functionalized with inclusion complexes demonstrated to be a viable alternative for the production of stable systems based on TML with potential for a safe and effective topical control release treatment.